Serologic detection of vaccine associate IgG responses in horses
using a multiplex magnetic microsphere assay

by

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Abstract

To protect horses from disease, equine practitioners typically prescribe a protocol of an initial primary vaccination followed by a booster vaccination 3-4 weeks later. Subsequent boosters are given every 6-12 months depending on the pathogen of concern. Each vaccination incurs an additional cost and increased chance for adverse reactions. Despite wide-spread protocol acceptance, duration of effectiveness of vaccines in protecting horses from disease is not well documented. It was hypothesized that horses vaccinated annually since birth have increased antibody production that remains consistent and sufficient for long-term protection from common diseases. This work resulted in the development of a novel, multiplex-magnetic bead-based indirect immunoassay to screen sera from vaccinated adult horses to measure antibody levels in response to vaccine administration. Antigens tested included West Nile Virus, Eastern Equine Encephalitis, Western Equine Encephalitis, Equine Influenza Virus, Equine Herpes Virus 1 and 4, Tetanus, and 7 different Rabies antigens (3 lab and 4 wild strains). The developed assay was a 7-plex capture antibody, which quantified equine IgG (Immunoglobulin G) that binds viral antigens derived from different rabies virus strains along with pure vaccine samples of the 7 different antigens. A 7-point standard curve was developed to quantify the viral-antigen reactive IgG concentration in vaccinated horse serum. Vaccinated horses increased serum antibody concentration for each antigen post-vaccination with the percent increase ranging between 34.0% for Equine Herpes Virus 4 and 257.3% for Equine Influenza Virus. Use of the novel assay will provide equine veterinarians with an economical method to measure immune activation toward common pathogens of concern. This methodology will provide foundation level information regarding antigen specific IgG concentrations that ultimately may be

extrapolated to establish protective levels of immunity resulting in establishment of vaccine

protocols.

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Acknowledgements

Dedication

This thesis is dedicated to my parents (Dave and Jill) who exposed me to the world of research and showed me the wonder it provides.

Chapter 1 - Development of a multiplex bead assay to determine antigen specific IgG induced by vaccination in horses

Equine vaccinations are traditionally given at 6-12 months old, followed by booster vaccinations every 6-12 months throughout the life span of individual animals (AAEP 2017). Vaccination programs are typically applied across all individuals based on existing protocols and exposure risk factors (AAEP 2017). Exposure risk factors include traveling, degree of contact with other horses, geographic location, level of activity, age, sex, and known viral outbreaks. To develop immunity to a disease, an initial vaccine series primes the immune system for action and booster vaccines induce a memory immune response (Davis 2014). Protection via the immune system in response to vaccination includes proliferation of antibodies and antigen-specific T lymphocytes capable of responding to a virus infection (Davis 2014). Of special interest to veterinarians and horse owners is the immune response affiliated with adverse vaccine reactions. The proliferation of lymphocytes can be used as an indicator of immune status from the vaccine reaction, allowing for the potential to identify extant immunity levels prior to vaccination of horses that exhibit adverse reactions to vaccinations administered on a predefined schedule (Kydd 2006). Development of a technique to quickly and accurately test immunity levels of horses that exhibit adverse reactions to booster vaccinations will allow owners the comfort of knowing their horses have safe levels of immunity without experiencing a potentially life threatening adverse reaction.

Viral vaccines contain viral antigens along with nontarget antigens including tissue culture-derived proteins and stabilizers (Gershwin 2012). The immune response to viral antigens typically also includes a mounted response to the nontarget antigens (Gershwin 2012). The specific response is IgE (Immunoglobulin E antibodies) to nontarget antigens, which has been

shown to put a patient at risk for developing Type 1 hypersensitivity as these antibodies cause an adverse or allergic reaction to the vaccine (Gershwin 2012). Systemic adverse reactions that have been documented in response to vaccine reactions range from urticaria to colic, and may be as severe as anaphylactic shock (Gershwin 2012). The major component of nontarget antigens is bovine serum albumin (BSA), which is used in vaccines for stabilization before administration. Horses with a less than 40% serum IgE response to BSA, but not having a two-fold increase post-vaccination do not show signs of hypersensitivity following vaccination; however, horses with a high measure of serum IgE in response to BSA typically have a response to vaccination with mild to severe adverse clinical signs (Gershwin 2012). From this information, it is speculated that horses who express IgE in this setting may be allergic to other vaccine components such as gelatin and casein.

There is a documented syndrome termed "ASIA" (autoimmune syndrome induced by adjuvants) that describes autoimmune/inflammatory syndrome specifically caused by adjuvants used in vaccines (Shoenfeld 2011). Adjuvants are used to boost an immune response in vaccines, with the effect accomplished by activating both the innate and adaptive immune systems (Shoenfeld 2011). Within the innate immunity, adjuvants augment responses by dendritic cells, lymphocytes, and macrophages to increase local reaction to antigens (Shoenfeld 2011). Adjuvants increase the protective and lasting immune response to the vaccine and may enhance vaccine efficacy (Shoenfeld 2011). In equine vaccines, common constituents include bovine serum albumin and aluminum, which can contribute to adverse reactions to vaccinations.

In addition, horses may be more likely to suffer an adverse vaccine reaction as they age because it has been suggested that IgE levels increase each year that a vaccine is given, which likely contributes to an increase in the saturation of mast cells that increases the likelihood of a

hypersensitivity reaction (Gershwin 2012). Older horses (>20 years old) have greater pre-vaccination circulating antibodies when compared to younger horses (4-12 years of age) (Muirhead 2009). Conversely, younger horses have a higher anamnestic response to vaccine administration (Muirhead 2009). For these reasons, Ryan (2014) suggested that it may be advantageous to vaccinate strategically through monitoring antibody levels rather than routinely administer booster vaccinations, which would help avoid serious and unnecessary adverse reactions.

The need for a rapid, economical, and user-friendly diagnostic test that provides veterinarians with antibody titers for numerous vaccines is necessary to limit hypersensitivity reactions due to vaccinations and provide options for development of individual-based strategies for vaccine protocols. A promising approach to test antibody titers for antigens produced in response to vaccination is the use of fluorescent bead-based multiplex assay. In order to establish the microsphere bead assay for clinical application, a standard must be established that will allow quantification of each individual analyte and therefore, its corresponding antigen. I established a standard curve that could be utilized for a defined amount of equine IgG to convert mean fluorescent intensity to mg/dL for a given amount of antibody that was measured. The primary objective for the standard curve was to develop a reliable constant that corresponded to the antigens being evaluated for that specific plate and can be utilized with every plate.

Material and Methods

Assay Development

Moore (2015) developed a multiplex-bead-based indirect immunoassay to screen sera obtained from vaccinated horses against rabies antigens isolated from 7 rabies virus isolates of skunks, bats, and lab strains. The technique utilized xMap technology, which employs

3

carboxylated magnetic microspheres that are distinguished by unique fluorescent spectral

properties. Amine groups on proteins (i.e., antibodies or viral proteins) were coupled by

carbodiimide chemistry to the xMap beads followed by incubation with test serum and then

equine specific biotin-labeled secondary antibody and a fluorescent reporter (Figure 1.1).

Blood samples were collected from 18 healthy adult horses on two separate occasions

(baseline before vaccination and 14 days following booster vaccination). Thirteen of the 18

horses had a history of prior rabies vaccination, but the other five samples were from horses with

an unknown rabies vaccine history. Following before-vaccination blood collection, horses were

vaccinated against rabies (Equirab, Merck). Two weeks following booster vaccination, additional

blood was collected in aseptic fashion. Approximately 10 mL of blood samples were collected

from the jugular vein following thorough cleaning using 70% isopropyl alcohol. Blood samples

were allowed to clot prior to centrifugation to obtain serum samples. Serum samples were

aliquoted into 2-mL vials and stored at -20º C. An individual 2-mL vial was thawed at room

temperature for each experiment to avoid multiple freeze thaw cycles and potential protein

denaturing.

Antigens provided for the project were laboratory-adapted strains of rabies viruses

included Challenge Virus Strain-11 (CVS-11), Flury-Low Egg Passage (Flury-LEP), and

Evelyn-Rokitnici-Abelseth (ERA). Wild type rabies strains included Eastern Pipistrelle,

Tadarida, South Central Skunk, and North Central Skunk, which were cultured from rabies-

infected brain tissue from animals that had been confirmed positive by Direct Fluorescent

Antibody test. Rabies virus strains were genotyped by PCR sequencing (Moore 2015).

Inactivation of the virus preparations were performed by serial passage of MNA or BHK cells

mixed with 0.5 mL of the inactivated virus suspension and microscopic examination on each day

of passage for virus infected cells using fluorescent conjugated anti-rabies antibodies. No virus was detected in the cultured cells (Moore 2015).

The coupling process was completed using Luminex bead coupling kit (xMAP Antibody Coupling Kit, Luminex, Austin, TX) to couple protein (antigens) to the microsphere beads. The microsphere analytes are carboxylated polystyrene microparticles, or "beads," that have been dyed into spectrally distinct sets, or "regions," allowing them to be individually identified by an xMAP® instrument (Baker 2012). The coupling process is a two-step carbodiimide coupling with sulfo-N-hydroxysulfosuccinimide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). The process was followed according to the manufacturer's instructions.

Each rabies strain was individually coupled to a specific bead, which was pre-determined based on the available regions that were available (Table 1.1). Selection of each specific bead was determined to provide a unique spectral analysis for each antigen allowing the assay to be applied in a multiplex manner (i.e., 12 for CVS-11); thus, anytime the instrument read region 12 that information would correlate with antigen CVS-11. Individual bead regions corresponded to emitted fluorescence detected by the Mag-Pix instrument.

Five µg of each antigen preparation was covalently coupled to 1.0×10^6 carboxylated paramagnetic microspheres (beads) (MagPlex microspheres, Luminex, Austin, TX). Confirmation of antigen binding was determined by testing the coupled beads with mouse anti-rabies glycoprotein (ABCam) and mouse anti-rabies nucleoprotein (Diagnostics/Millipore), and detected with R-PE-goat anti-mouse IgG (H+L) 2 µg/mL (Columbia Biosciences) (Moore 2015).

The assay was performed on a MagPix instrument (Luminex, Austin, TX) using a three-stop process (Figure 1.2). For each assay, a master mix of the analytes (beads) was made based

on how many antigens were being tested and how many serum samples were being evaluated. An equal volume containing 100 beads (using the formula C1V1=C2V2) of each specific analyte were mixed together with phosphate buffered saline (PBS) and 50 µLs were aliquoted to each well. Serum samples were serially diluted using PBS (1:125, 1:500, 1:2000, and 1:8000) to best determine the most useful dilution. The assay was created by combining 50 µLs of each serum dilution with 50 µL of the bead mix (2.5 X 106 beads/mL) in wells of a 96-well reaction plate, incubated overnight in the dark at 4º C on a shaker (200 rpm). After the serum was been added, a wash process was initiated that utilized the magnetic nature of the analytes. The 96-well plate was placed on a magnetic separator for two minutes, which allowed for the magnetic analytes to migrate to the bottom of the well. The excess fluid was removed and analytes were washed twice with 50 µLs of PBS-Tween 2%, which decreased non-specific background binding. Once washing was complete, the second step was to add a secondary anti-body, which was Biotin Goat anti-Horse IgG at 2.0 µg/mL (Columbia Biosciences). The secondary master mix was determined by the number of wells being utilized on the 96-well plate (C1V1=C2V2) and the concentration was made using PBS. Then 50 µLs of the master mix at the correct concentration was placed in each well. The plate was again incubated in the dark at 4º C on a shaker (200 rpm) for two hours. After two hours, the wash process was completed again. The third step was adding PE-Streptavidin, which is a fluorescent label at a concentration of 4.0 µg/mL (Jackson Lab). The mast mix was made the same way as previously described, then 50 µLs was added to each well. The plate was incubated in the process for one hour, and two washes were completed again. The final step added 50 µLs of the driver fluid (provided by the MagPix instrument) to each well allowing for thorough mixing, and the plate was placed in the instrument for running.

The MagPix instrument utilizes two LED lights where the red LED light excited the fluorescent dyes in the bead itself and green light excited the fluorescent reporter (streptavidin-Phycoerythrin) that was added in step three. The MagPix instrument expressed these data as mean or median fluorescent intensity.

Standard Curve

Development of a standard curve required that each antigen coupled to microsphere bead have a corresponding standard analyte (have the same regional number that is what is read by the MagPix instrument). For an individual analyte, a specific area is fluorescent to express a region that had a corresponding number assigned to it (e.g., 15). For example, an analyte will have region 15 fluorescent. Region 15 was pre-determined to have the CVS-11 antigen coupled to the bead (described earlier in this chapter). The standard curve would additionally have the analyte with region 15 fluorescent; the difference would be that this antigen would be the known amount of equine IgG. Each of the seven vaccine antigens (results in Chapter 2) and seven rabies antigens (this Chapter) coupled to a microsphere analyte for the vaccine assay would have a corresponding standard curve microsphere analyte (i.e., there would be two region 12 beads where one was coupled with the CVS-11 antigen and the corresponding bead would have the known amount of equine IgG).

The goal of the standard curve for the assay is to utilize a standard to be available for results from any equine sample tested to be computed in a graph or standard range to determine if titers of the patient are considered in the range of normal, healthy horses with a known vaccine history. The standard curve will allow for a mechanism for comparing antibody responses among each equine serum samples and potential of determining if the equine sample is at a protective level with its antibody production.

I followed methods outlined earlier in this chapter for coupling the known amount of equine IgG to the microsphere analytes (Figures 1.1 and 1.3). The same process of running the assay as described above was utilized for the standard curve. Documented differences included that each standard analyte was coupled with 25 µg of purified mouse anti-human IgG (BD Biosciences) via a carboxiide reaction using the Luminex protocol that was provided (previously described in Chapter I). Each region that was used was coupled with 1 million microsphere beads (compared to vaccine antigens that were coupled with 2.5 million microsphere beads). An additional difference observed for the standard curve was for step 2, where there was a 7 dilution series of the horse reference serum with known amount of IgG. The dilution series began with 1:1.56 – 1:100, and the dilution series was pipetted into the 96-well plate in duplicates.

Results

For rabies antigen results, data generated following vaccination revealed a non-uniform pattern of antibody production by individual horses against various rabies strains (wild and laboratory strains) (Moore 2015). All horses increased their antibody production following vaccination against all rabies strains that were tested (wild and laboratory strains). The process successfully captured variation among individual horses. For example, an individual horse, Neitto, had a low antibody response pre and post vaccination (<965 and 1,743 MFIs) suggesting that he had never been vaccinated against rabies prior to the present study (Figures 1.4 and 1.5). When comparing the wild-type strains to the laboratory strains, antibody production against the wild-type stain had 5-10 fold less IgG production when compared to laboratory-adapted rabies strains (Figures 1.6). Ten horses had a greater than 2-fold increase in antibody response to the rabies laboratory vaccine strains with seven of the horses had the highest antibody response to

the ERA strain (Figure 1.6). The additional eight horses had a one to two fold increase to the wild-type strain, but a specific strain was not predominate. (Figure 1.6)

In a clinical application, the mean fluorescent intensity was converted to milligrams per deciliter (mg/dL) using Milliport xPonent Software when comparing the results to the standard curve. These results use the mean from a paired t-test of all 18 horses for each of the seven different rabies strains from Moore (2015). Comparison of average antibody levels in response to seven rabies strains in 13 horses vaccinated using rabies vaccine (Merck). Post-vaccination antibody levels were greater than pre-vaccination for all rabies types (* indicates a statistical difference [$P < 0.05$]) with % increasing being a range of 62% for SCS and up to 602% for E. Pip (Figure 1.7). The relative low values for the North Central Skunk (NCS) and South Central Skunk (SCS) results were attributed to poor passage when growing the strains and not to decreased immune response in the horse. The three strains highlighted in purple in Figure 1.7 are the lab strains of CVS-11, ERA, and Flury; the remaining strains were wild type Eastern Piperselle and Tadaria. The vaccine that was administered to our study group was most closely related to ERA.

In addition, the assay was applied to a clinical setting to determine individual IgG responses in an individual horse who had a history of an adverse reaction post rabies vaccination and another individual with an unknown vaccine history. For comparison purposes, these horses were compared with H1 who was a Kansas State University owned horse with a known history of annual booster vaccines and therefore, served as a control. H2 was the horse with an unknown vaccine history. H3 was a horse with adverse vaccine reaction following a rabies booster. H1 had an amnestic response to all of the rabies antigens (lab strains and wild-type strains). H2 did not demonstrate IgG production toward rabies antigens. H3 was recently vaccinated and

demonstrated a marked humoral and amnestic response (Figure 1.8). Although the horse had an

adverse reaction to vaccination, this individual demonstrated an appropriate IgG response to

various rabies antigens.

Standard Curve

The standard curve was established as part of every plate analyzed during this project.

The standard curve was not consistent for this project, but I was able to establish that there was

successful coupling of the mouse anti-human IgG to each analyte as there was an increase in

mean fluorescent intensity for each increase in concentration (Figure 1.9). In addition, I was able

to determine the most successful concentration of horse reference serum was 1:2000. In addition,

the curve allowed for each individual equine patient sample to be compared to a constant (Figure

1.10).

Read outs that were provided using the Milliport xPonet Software provided a lower limit

of detection and an upper limit of detection with the 7 series of dilutions of the standard curve

between them (Figures 1.10 and 1.11). This range allows for each individual equine patient

sample to be compared to a constant, and then compared to each other. As an example of a read

out from MilliPort Software for one rabies analyte (Flury): from left to right the columns are -

location on the 96-well plate, the expected ng/mL is the standard dilution factor, the mean

fluorescent intensity for each individual well, the ng/mL is the MFI converted to ng/mL, the next

set of MFI and ng/mL is the average of the previous values, CV (coefficient of variation) is the

% of replication between the duplicates for each individual dilution factor (want >90%), and

recovery is the percentage that the machine was able to recover from each well (want 100%)

(Figure 1.12). An example of a read out from the MilliPort Software is provided for equine

serum samples that converts the average of the duplicates from mean fluorescent intensity to a

clinical significant concentration where the software then compares the value to the standard curve, and expresses the value if below the lower limit of detection (black), if within the limit of detection (blue), or if above the upper limit of detection (red) (Figure 1.13). I found that the standard curve was inconsistent in the range with numerous variables that affected it.

Discussion

Rabies is prevalent in the environment throughout the United States with primary reservoirs differing based on location (i.e., skunks in the midwest or raccoons on the east coast). Reports from Centers for Disease Control, wild animals account for 92.6% of reported cases of rabies (Moore 2015). With skunks being the primary reservoir within the midwest, there are other reservoirs that are becoming more apparent, particularly as more research is completed to investigate various bat strains. As a result, the risk of exposure to horses to wild strains of rabies is increasing. Thus, the primary aim of this study was to determine antibody cross reactivity of equine IgG following vaccination to include not only laboratory adapted strains, but also wild type strains.

The primary objective of previous work (Moore 2015) was to develop a microsphere assay capable of determining if horses vaccinated with a commercial rabies vaccine produced cross reactive antibodies against the G protein of laboratory and wild strains of rabies. This assay was paramount and foundational for the currently reported investigations. Results from this primary investigation revealed that vaccinated horses produced cross reacting antibodies against the wild type antigens. Further, results revealed that subjects produces a higher antibody titer to the laboratory adapted strains of rabies that are used in vaccine production when compared to antibody titers against wild rabies strains. The assay allows for a high throughput using a small blood sample.

The current vaccine used for protection from equine rabies was based on one of the three types of laboratory strains that were passed on from Pasteur's strains. As more strains of rabies are identified in nature, the question has been raised whether horses are produce cross reactive antibodies following routine vaccination. With the developed micro-sphere assay, Moore (2015) demonstrated that the immune response following vaccination differs greatly from horse to horse. This was attributed to variability of IgG production among individual horses following vaccination. In addition, results from the assay were able to verify that horses are capable of producing antibodies to numerous strains of rabies; this can be due to environmental exposure or from the vaccine booster itself. It is encouraging that the horses produced antibodies to all 7 strains of rabies tested.

Subsequent to the rabies vaccine investigation, the assay was extrapolated to determine IgG responses against equine vaccine antigens including West Nile Virus, Eastern Equine Encephalitis, Western Equine Encephalitis, Tetanus, Equine Herpes Virus – 1, and Equine Herpes Virus – 4 (Chapter 2) using similar methods as described in this chapter. The primary objective of the subsequent investigation was to establish an assay for routine clinical work to determining antibody response to numerous antigens.

Although I was able to develop a standard curve, unfortunately, there remains some uncertainty in the potential application of the curve. I consider these results to be experimental and preliminary, suggesting further work to conclusively establish a standard curve. I did confirm the binding of mouse anti-human IgG to the microsphere beads. I also confirmed that with the curve and patient serums, the equine serum antibody response fit when compared to a known amount of equine IgG. These data allowed me to quantify if patient's antibody levels were considered in danger, normal, or reactive towards a specific antigen. Future studies for

development of the standard curve need to explore how to make the calculations of dilutions of the horse reference serum more consistent, the longevity of the bead's life, and consistent data with patient sample.

Table 1.1- Visual representation of pre-determining rabies antigens

RABIES ANTIGENS

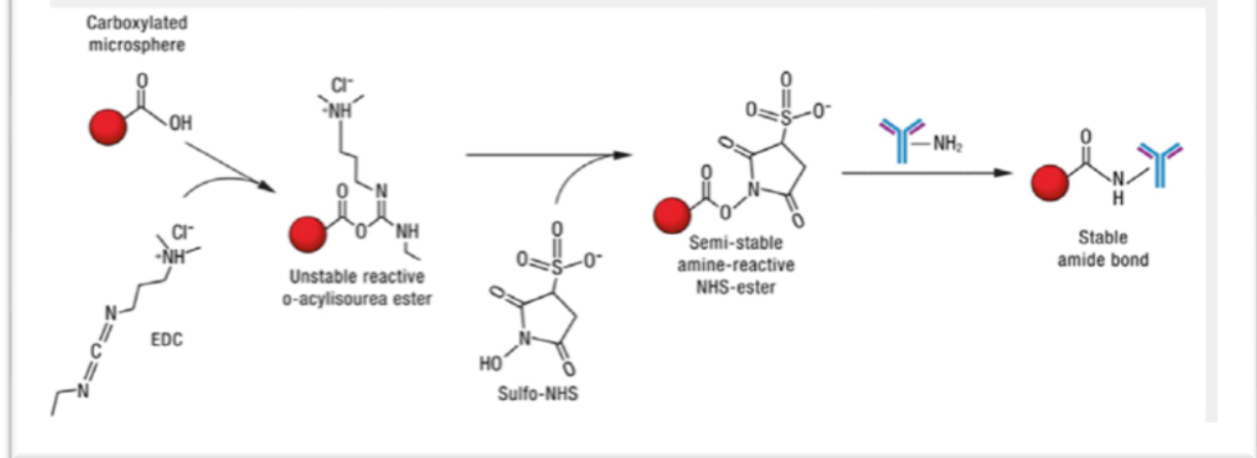| Analyte	12	15	20	25	28	34	42	48	53	56	62	66	74
CVS-11		X											
E. pip						X							
NCS								X					
Tad									X				
SCS										X			
ERA											X		
Flury												X	

Figure 1.1: A representation of the coupling process when starting with a carboxylated microsphere and through two reactions with a specific antigen, the microsphere ends the coupling process with a stable amide bond.
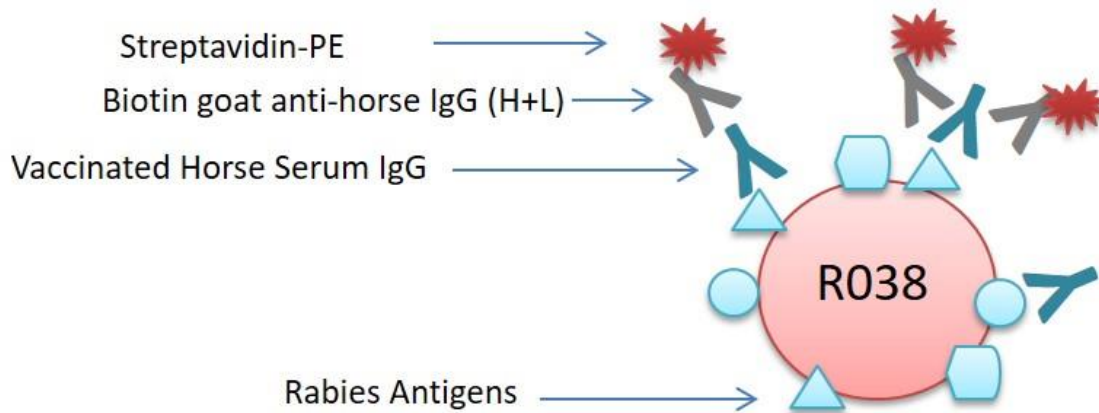
Figure 1.2 – Visual representation of the coupling process using one rabies antigen and the three steps that follow. (Figure provided by Kelley Black, Kansas State University)
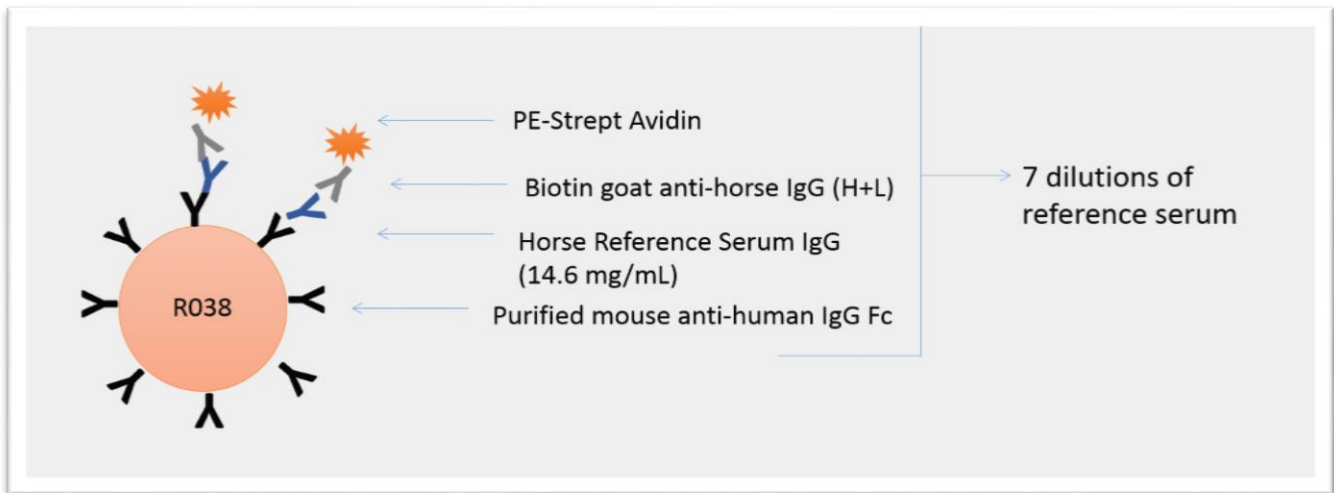
Figure 1.3 - A representation of the end result of the coupling process for a capture antibody assay (standard curve). (Figure provided by Kaori Knights, Kansas State University).

Figure 1.4 - Mean fluorescent intensity readings for 18 different horses pre-vaccination to both the lab-adapted strains (CVS-11, ERA, and Flury) and the wild-type strains (E.Pip, NCS, SCS, and Tad)

Raw data (MFI): Equine IgG Rabies Antigens After Booster Vaccination
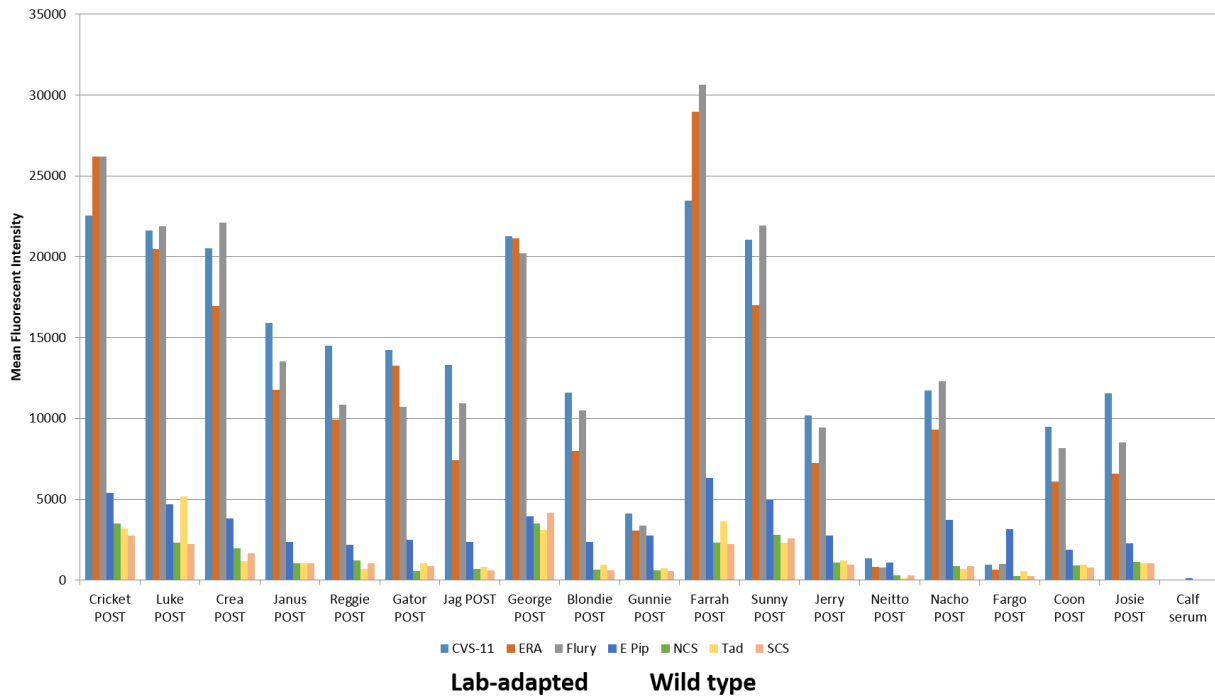
Figure 1.5 - Mean fluorescent intensity readings for 18 different horses post-vaccination to both the lab-adapted rabies strains (CVS-11, ERA, and Flury) and the wild-type strains (E.Pip, NCS, SCS, and Tad)

Raw data (MFI): Fold Increase of Equine IgG against Rabies Antigens After Vaccination
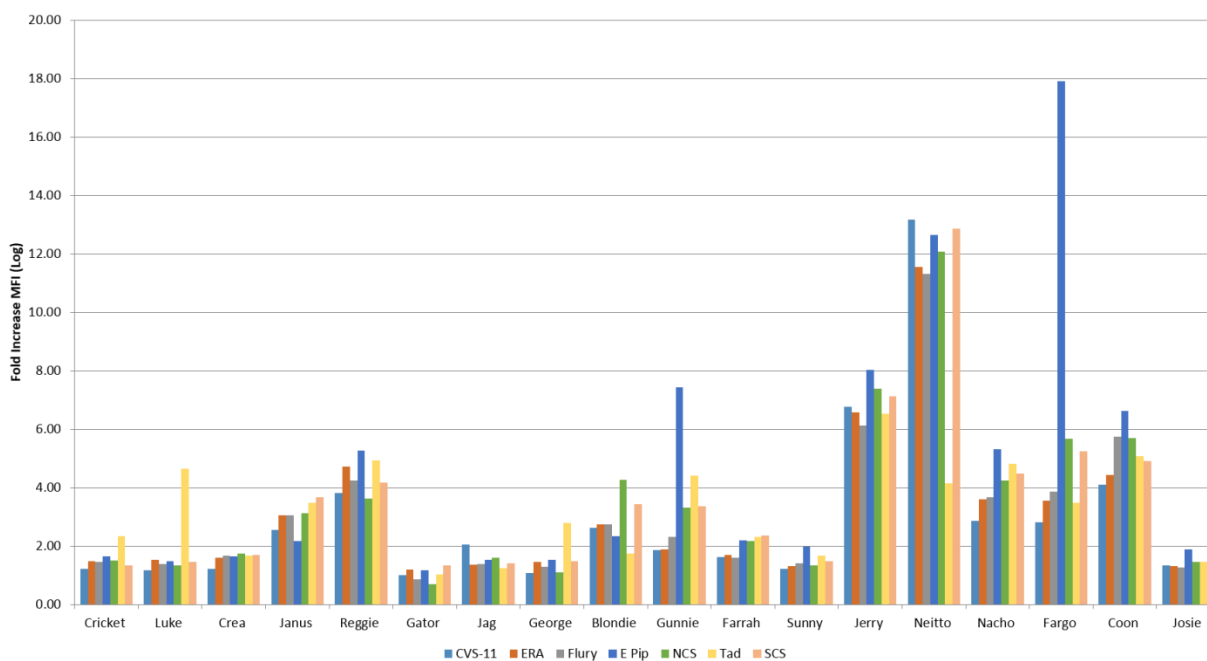
Figure 1.6 - Mean fluorescent intensity fold increase for all 18 horse samples post-vaccination

for all 7 strains for rabies (CVS-11, ERA, Flury, E.Pip, NCS, Tad, and SCS)

IgG concentration (mg/dL) against Rabies Antigens
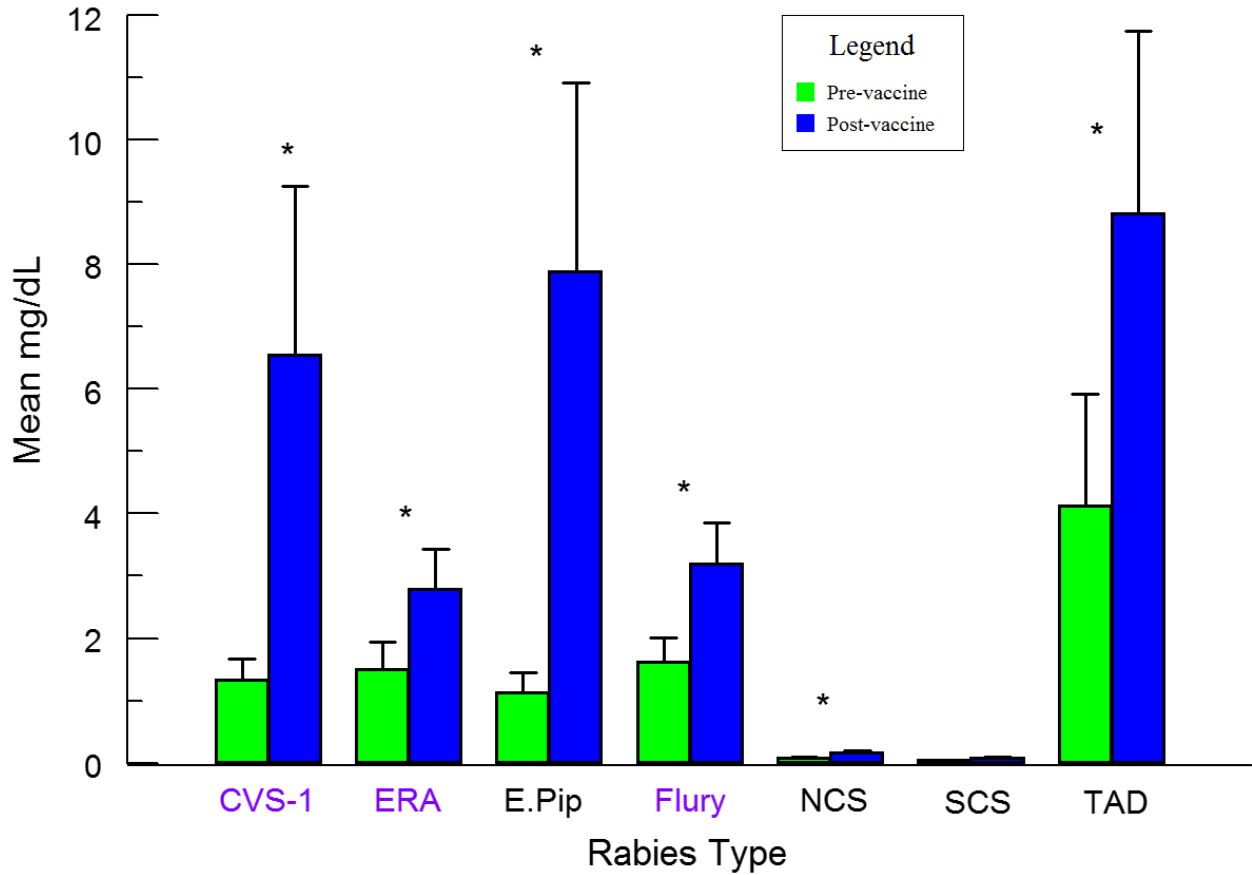
Figure 1.7 - Clinical representation using milligrams/deciliter units for all 7 rabies strain (lab-adapted strains in purple font (CVS-11, ERA, Flury) and wild-type strains in black font (E.Pip, NSC, SCS, and Tad))
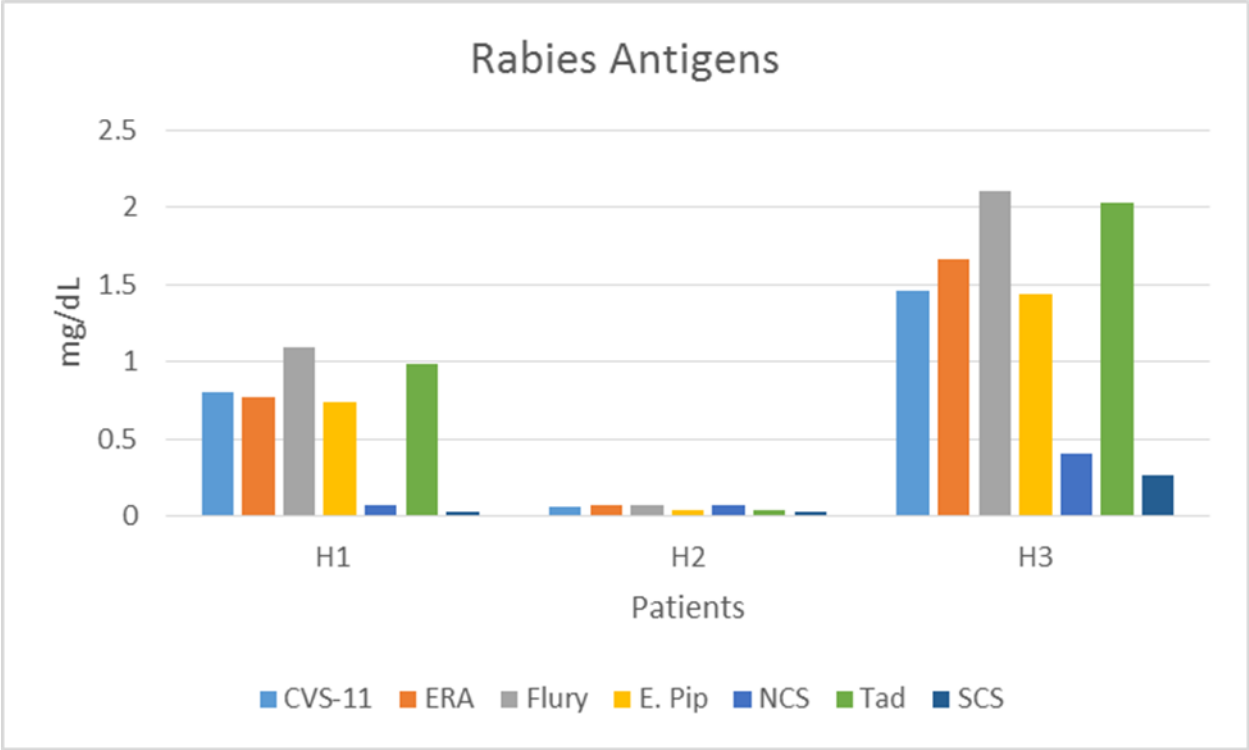
Figure 1.8 - Graphical representation of clinical significant results with horse H1 being a constant, H2 has an un-known vaccine history, and H3 has a history of a vaccine reaction to a rabies vaccine.
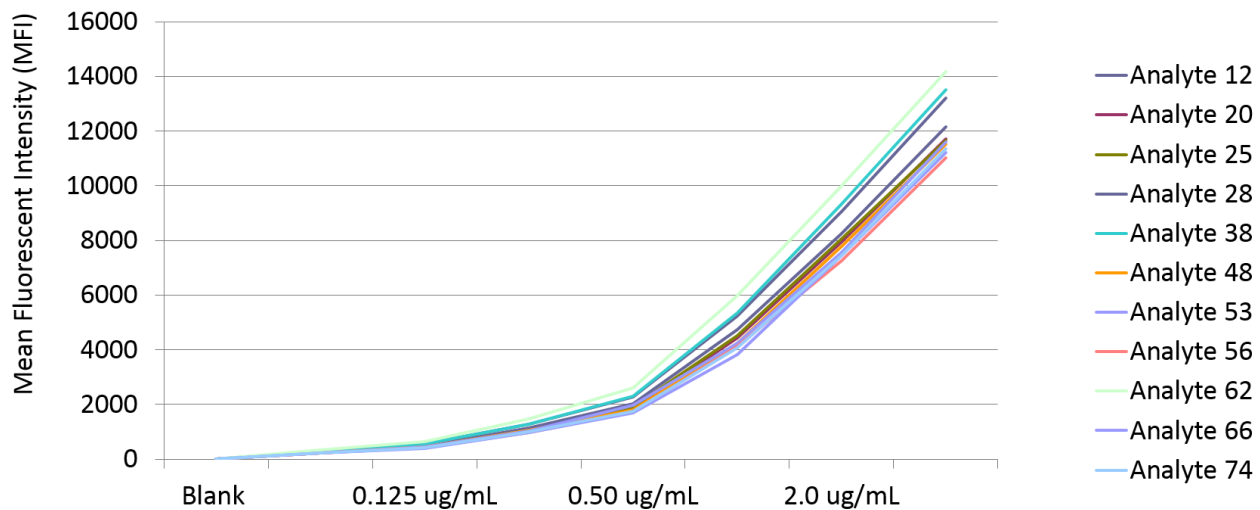
Figure 1.9 - Graphical representation of successful coupling of mouse anti-human IgG to each analyte.
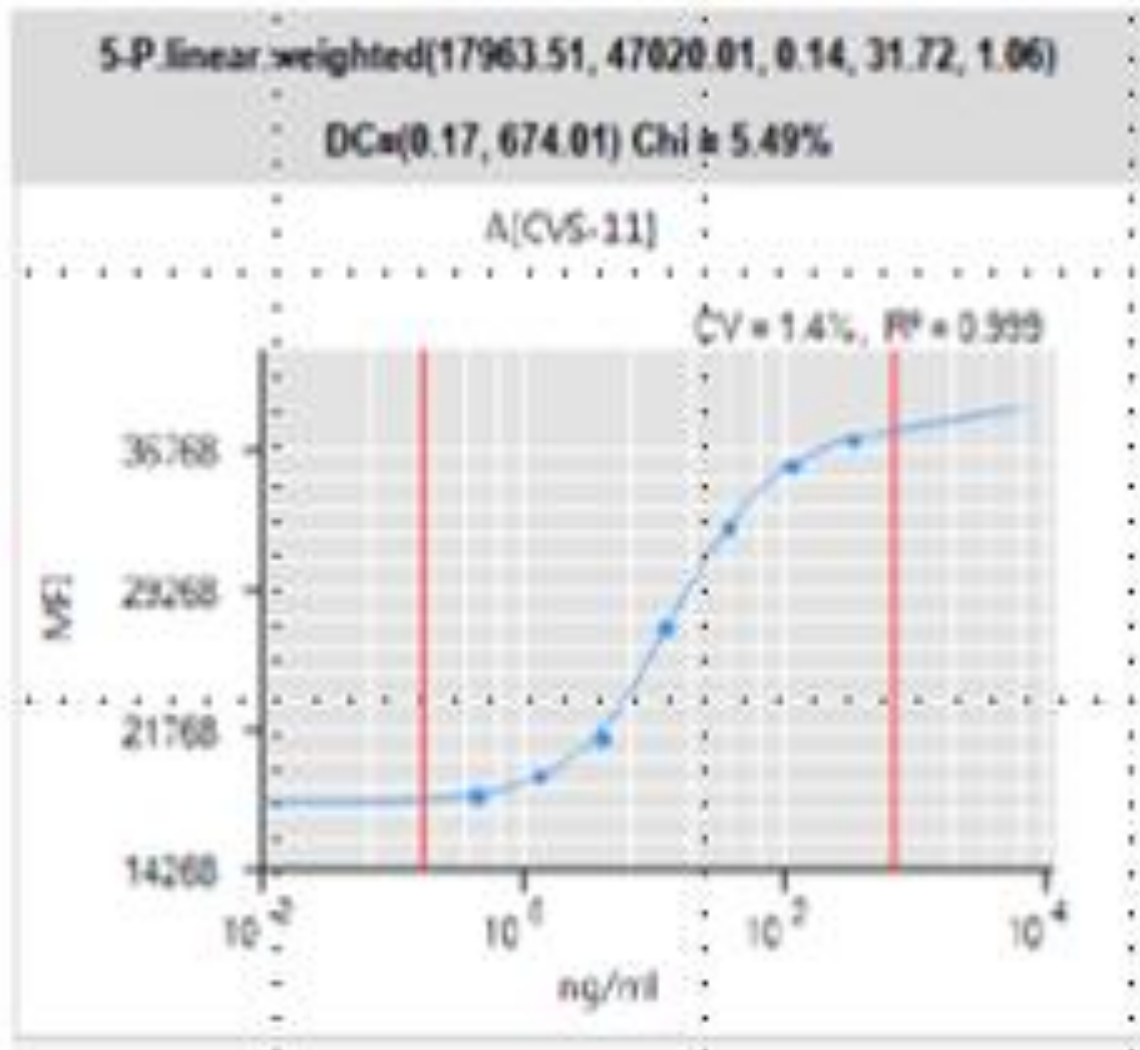
Figure 1.10 - Single representation of a standard curve that correlates to antigen CVS-11 and is converting mean fluorescent intensity to nanograms/milliliter
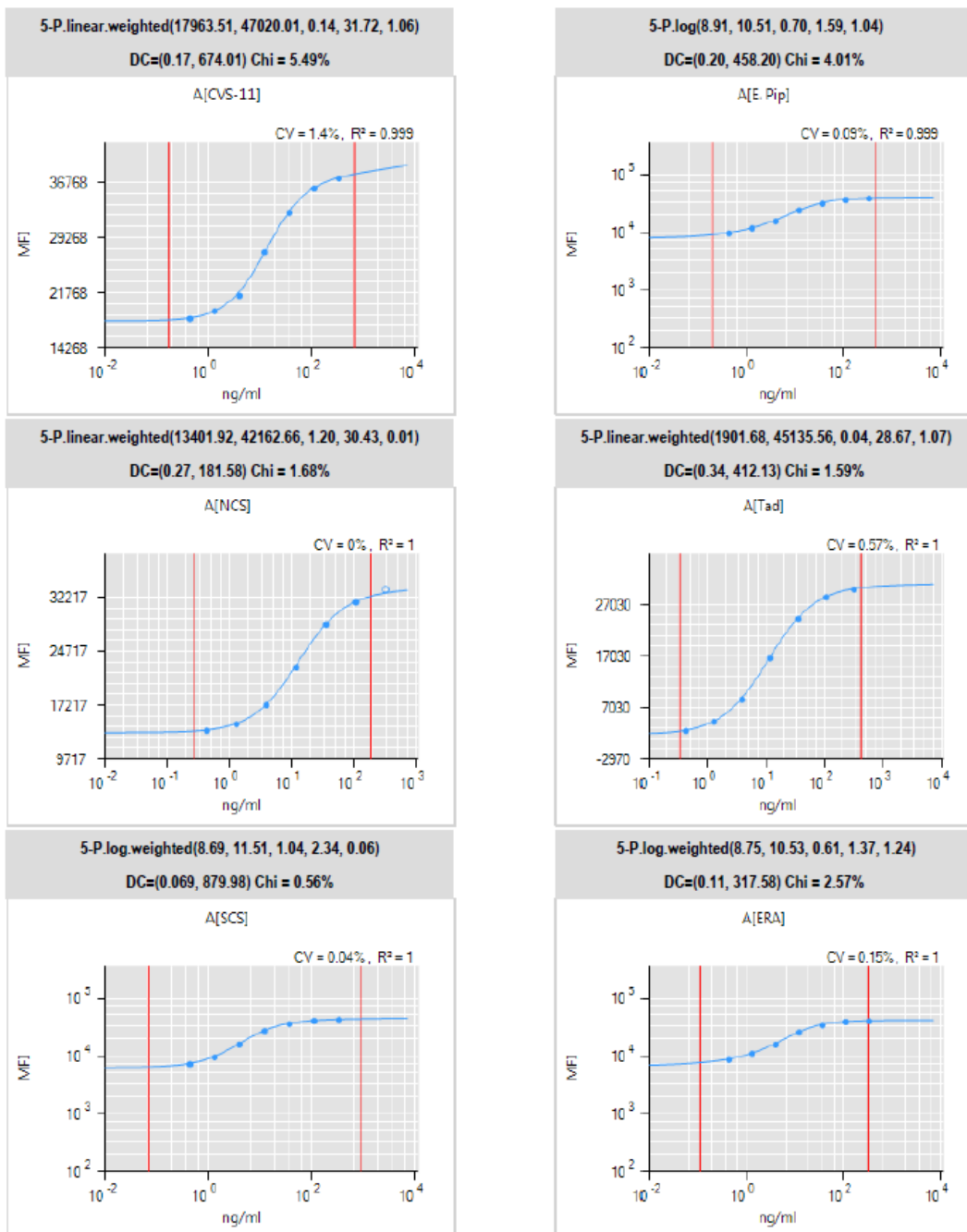
Figure 1.11 - Visual representation of six separate standard beads and the graphs converting the mean fluorescent intensity to nanograms/milliliter

MILLIPLEX Analyst Detail Report

Analyte Flury

5-P.linear.weighted(983.86, 57383.76, 0.04, 56.50, 0.90)

Chi=7.76%, CV=3.31%, R²=0.994, DC=(2.63, 761.82)

| Location	Expected ng/ml(i)	MFI(i)	ng/ml(i)	MFI	ng/ml	CV	Recovery
1A3	0	6317		6359		0.94%	
1B3		6402					
1C3	7.81	12978	7.79	12903	7.71	0.82%	98.71%
1D3		12828	7.63				
1E3	15.62	17685	13.78	19552	17.06	13.5%	109.2%
1F3		21419	21.1				
1G3	31.25	23338	26.35	23194	25.91	0.88%	82.91%
1H3		23050	25.47				
1A4	62.5	30734	72.42	30198	66.22	2.51%	105.95%
1B4		29662	60.79				
1C4	125	33377	122.28	33558	127.67	0.76%	102.13%
1D4		33739	133.45				
1E4	250	35924	265.72	35725	245.42	0.79%	98.17%
1F4		35526	227.72				
1G4	500	36515	348.19	37125	497.7	2.32%	99.54%
1H4		37734	823.22				
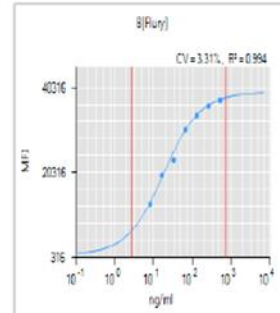
Notes:

CV-The Coefficient of Variation of standard curve replicates at each dilution level.

Chi-The Chi-Square test statistic of the distance between observed concentrations with expected concentrations.

Figure 1.12 - Visual representation of the data that correlates to the graphical representation that is converting mean fluorescent intensity to a clinical significant concentration.

MILLIPLEX Analyst Detail Report

Samples:

Location	Sample	MFI(i)	ng/ml(i)	MFI	ng/ml	CV
1A5	1A5	33	<2.63↓	33	<2.63↓	0%
1B5	1B5	37	<2.63↓	37	<2.63↓	0%
1C5	Nacho Pre(200)	11594	6.45	9632	962.19	28.82%
1D5		7669	3.4			
1E5	Nacho Pre(1000)	5806	<2634↓	5531	<2634↓	7.04%
1F5		5256	<2634↓			
1G5	Nacho Post(200)	13291	8.11	13052	1572	2.59%
1H5		12813	7.62			
1A6	Nacho Post(1000)	19055	16.12	18250	14705	6.23%
1B6		17446	13.41			
1C6	Fargo Post(1000)	797	<2634↓	750	<2634↓	8.86%
1D6		703	<2634↓			
1E6	Fargo Post(200)	1447	<526.78↓	1840	<526.78↓	30.18%
1F6		2232	<526.78↓			
1G6	Fargo Pre	787	<526.78↓	697.75	<526.78↓	18.09%
1H6		608.5	<526.78↓			

Notes:
Red-Higher than maxDC, Black-Lower than minDC, Blue-between maxDC and minDC;
NAN-Curve Fitting Failed; N/A-Data marked out; ND-missing original data

Figure 1.13- A visual representation of the results in duplicates of two equine serum samples (Nacho and Fargo) being converted from mean fluorescent intensity to a clinical significant concentration (ng/ml)

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Chapter 2 - Determination of Vaccine Antigen Specific IgG Expression Following Vaccination Using a Multiplex Microsphere-Based Assay

Introduction:

Equine vaccination is a routine component of preventative health care for horses. Vaccine protocols are typically initiated between 4 and 6 months of age. Equine vaccination protocols are based on the recommendations made by the American Association of Equine Practitioners (AAEP) Biological and Therapeutic Agents Committee. The AAEP recommends that all horses annually receive a set of core vaccines, which are West Nile Virus, Eastern Equine Encephalitis, Western Equine Encephalitis, Tetanus, and Rabies. In addition, there are numerous risk-based vaccines for additional diseases that can be given to the horse based on where it is traveling, if there is a potential for exposure, or an outbreak has occurred (Balaysuria 2017). Veterinarians are constantly questioned whether there is a need to vaccinate individual horses for all pathogens. An added common question includes, why a horse should require rabies vaccination annually when canine vaccines protocols are for every 3 years (following initial series) (Harvey 2016). One reason that horse owners are reluctant to implement extensive vaccine programs involves an ever growing concern for potential adverse reactions that may be directly related to vaccinations.

Vaccine reactions can occur under a variety of settings. Reported adverse reactions range from swelling at the injection site, swelling and stiffness through the neck, or urticaria. (Gershwin 2012). Other concerns of owners involve the cost of vaccines, and owners questioning why their horse needs to receive booster with vaccination every 6-12 months. Normally, the

vaccine protocol for a horse includes a 5-way vaccine that protects against Influenza, Tetanus, Eastern Equine Encephalitis, Western Equine Encephalitis, and West Nile Virus along with annual vaccination for Rabies. When a horse has a vaccine reaction, it is hard to determine which specific antigen caused the reaction as the vaccine itself contains up to five different antigens and usually given with a Rabies vaccination. Typically, veterinarians give the 5-way vaccination and Rabies on different sides of neck, which allows veterinarians to narrow down causes of injection site reactions.

Having the ability to use a serum sample and a rapid, inexpensive laboratory test to determine which vaccine component is causing the reaction will help determine subsequent vaccine recommendations. The opportunity for veterinarians to take a serum sample from a horse and know the titer values for up to 14 different vaccines is immensely helpful for development of vaccination protocols for individual horses. Veterinarians would be able to follow titer patterns on an individual horse, determine which antigen the horse is having a reaction to, or save their clients' money if the titers are considered within normal limits for the antigen with an assay system that provides a cost effect option along with the ability to have high throughput.

Multiplex bead assay technology previously reported by Moore (2015) demonstrated that a currently applied rabies vaccination in the equine industry was able to elicit an antibody response against wild-type strain rabies found in the environment (Chapter I). It was the aim of this investigation to determine if this technology could be applied to other vaccine antigens. Zoetis generously provided 7 different antigens from their vaccine set for me to perform this assay. These antigens included: West Nile Virus (WNV), Easter Equine Encephalomyelitis (EEE), Western Equine Encephalomyelitis (WEE), Tetanus antigen, Influenza antigen, Equine Herpes Virus 1 and 4. The primary objective was to create an assay that would detect changes in

antibody expression in response to vaccination among healthy horses with known vaccine history.

Material and Methods

Twenty two adult horses were used in the study. Ten horses from the Kansas State University teaching herd while twelve were privately owned. Three additional horses were included based on having adverse vaccine reactions. Twenty one of the twenty two horses had a history of annual vaccinations. Blood samples were collected on the day of vaccination as described in Chapter I. Blood samples were stored at -20° C after clotting and serum being aliquoted.

Seven different inactivated vaccine antigens were provided by Zoetis and seven different rabies antigens provided from a previous study (Chapter I, Moore 2015) were also used with the assay development. The coupling process of the antigens to the beads was as previously described in Chapter 1, but at this time each individual antigen was bound using 20 µg of each virus. To confirm antigen coupling to the beads, the beads were incubated with an anti-antigen (for example WNV or Tetanus). (Table 2.1) There was successful coupling for West Nile Virus and Tetanus as the Mag-Pix instrument provided mean fluorescent intensity.

Previous experiments that were described previously used the same indirect immunoassay as performed for the rabies antigens (Moore 2015). All assays were performed on a Mag-Pix instrument (Luminex) and measured in mean fluorescent intensity. The same process of coupling and steps of assay were the same as previously described in Chapter 1. The incubation periods between each step did change. The first and second incubation (after adding

equine serum and secondary anti-body) was at room temperature on a plate shaker (200 rpm) for one hour, and the last incubation with the fluorescent tag was only for 30 minutes.

For assay development, an intra-assay and inter-assay were performed and calculated using coefficient of variation (mean of the population). The intra-assay assessed two equine samples in duplicates on the same 96-well plate in the first column and the last column. The optimal coefficient of variation for an intra-assay is <10%; all of results reached that optimal percentage. The inter-assay used the same equine samples in duplicates on two different plates at two different run times. The optimal coefficient of variation is <30% for the inter-assay. For the vaccine antigens, both horse one and two were below optimal coefficient of variation, but horse 2 for the rabies antigens was above the optimal coefficient of variation. (Figures 2.1 and 2.2)

Results

Horses were vaccinated in routine fashion and demonstrated a significant increase in vaccine antigen specific IgG responses post vaccination (Figure 2.3). Of note, the most robust amnestic response was observed following vaccination with tetanus and the lowest amnestic response was to EIV. The pre-vaccination graph has the highest limit of the mean fluorescent intensity of 40,000 while the post-vaccination graph reaches up to 50,000. Statistical analysis of the results was non-parametric data and used Wilcoxin Signed Rank test, and the results were that all seven antigens were significant ($P < 0.05$) post vaccination (Figure 2.4). The percentage differences for all 22 horse pre to post vaccination were EEE – 102%, EHV-1 – 52%, EHV-4 – 93%, EIV – 78%, TET – 310%, WEE – 84%, and WNV- 69%.

In a clinical application, the assay was used to evaluate clinical relevant equine samples where one had an unknown vaccine history and the other had had a vaccine reaction

post rabies vaccination and a third equine sample with a known vaccine history was added as a control. Horse 1 (H1) was a control that had a known vaccine history and was boostered yearly with titers that were considered within normal limits. Horse 2 (H2) was a horse with an unknown history of vaccination. H3 was a horse with a demonstrated adverse vaccine reaction and the owners were considering foregoing vaccinations if the antibody titers were within normal limits. H1 has an amnestic immune response to all antigens demonstrating a previous vaccine booster history. H2 has a low amnestic response, and does not appear to have been vaccinated for any of the vaccines. Recommendation for H2 would be a vaccine with a booster in two to three weeks. H3 has a decreased amnestic response when compared to H1, and recommendation would be booster of all antigens. As this is the horse who had a previous vaccine history, precautions would be recommend such as administering flunxin meglumine before vaccination or to spread vaccines out over two to three weeks.

Discussion

Within the literature, it has been suggested that the focus of the equine industry should be transitioning to strategic vaccinating over routine vaccinating (Muirhead 2009) For this to become a reality, an assay needs to be developed that was economical, high throughput, and user friendly (Baker 2012). The assay developed using microsphere beads will allow for up to 30 samples to be tested at once, with results within 8 hours. With the establishment of the standard curve (Chapter II), this provides a constant for each batch that is completed allowing for congruency for the assay.

Another main focus is increasing reports of vaccine reactions (Ryan 2014). For horses exhibiting a reaction post-vaccination, this assay would allow for a small volume of serum to be assessed whereby the resulting titers can be compared to the standard curve to determine which

vaccine is causing the reaction. In addition, pre-vaccination titers can be evaluated and when compared against the standard curve, a strategic vaccine plan can be developed. Vaccine reactions can be due to adjuvants that are in a vaccine along with the specific antigens (Gershwin 2012)); thus, more work needs to be developed to look into IgE responses to the adjuvants that are commonly used in equine vaccines.

Conclusion

.   The assay is novel and has the potential of providing beneficial information regarding antibiotic titers to veterinarians to improve treatment of horses subject to vaccine reactions. In addition, this assay will assist with the development of further understanding of effective levels of vaccines and duration of vaccine effects. Future work for the assay includes testing functionality of the antibodies along with quantity, establishing protective level values for each antigen, and establishing more data that involves immune-compromised horses or horses that have had a history of vaccine reactions.

Table 2.1 – MFI readings of anti-WNV and anti-tetanus against antigen

coated analytes

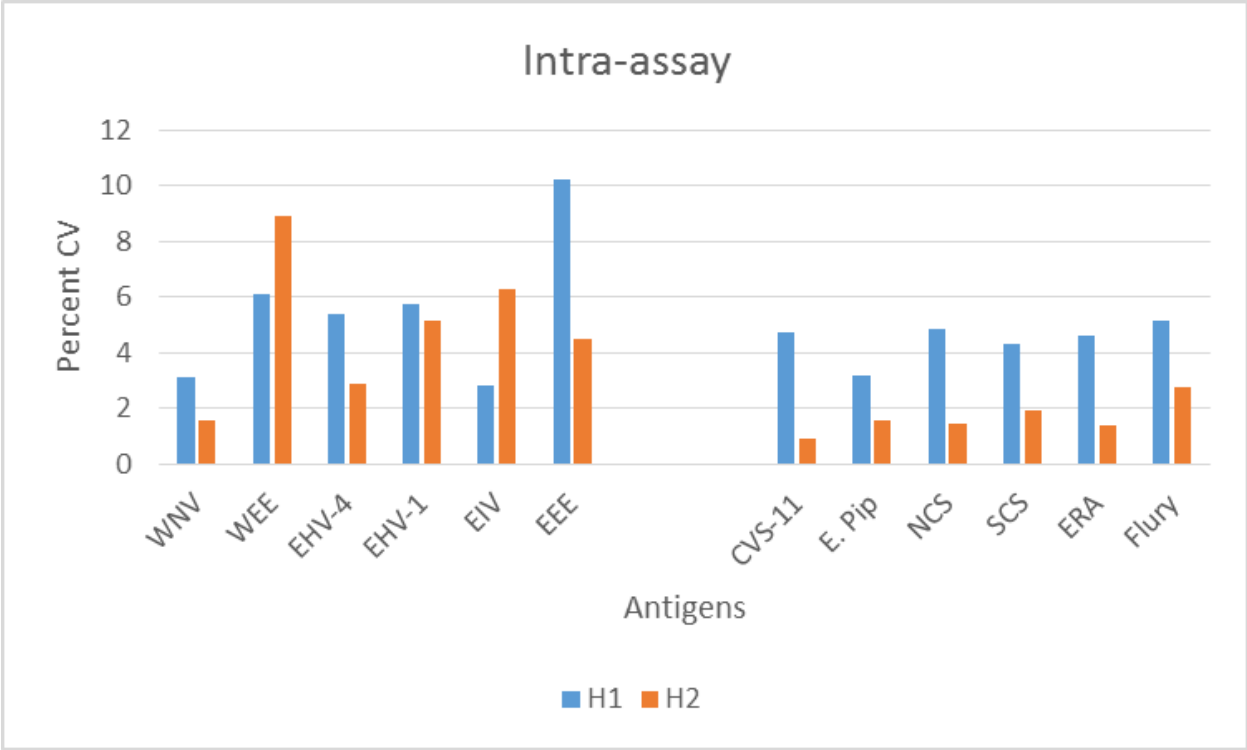| Analyte	15 (Tetanus Toxoid)	12 (WNV)
Anti - WNV		41 MFI
Anti-Tetanus	449 MFI	

Figure 2.1 – Intra-assay using coefficient of variation to determine accuracy of the assay within
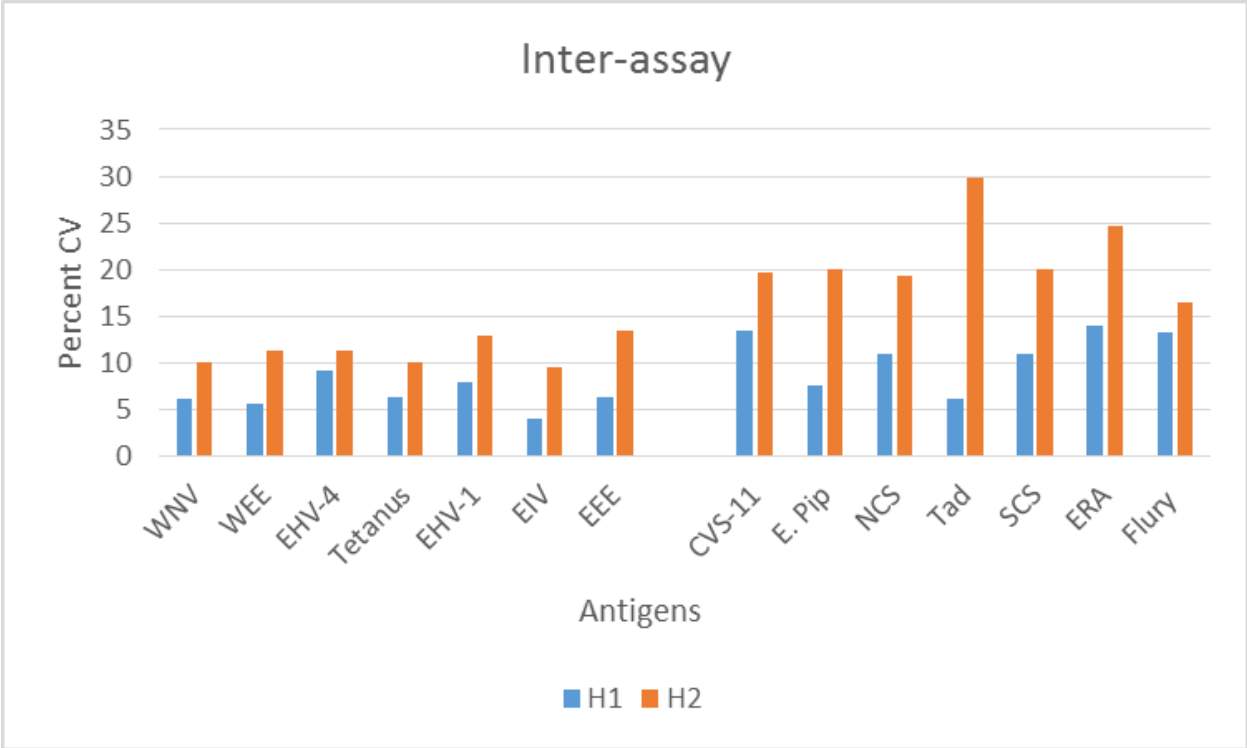
a single plate.

Figure 2.2 – Inter-assay using percent of coefficient of variation looking at accuracy between
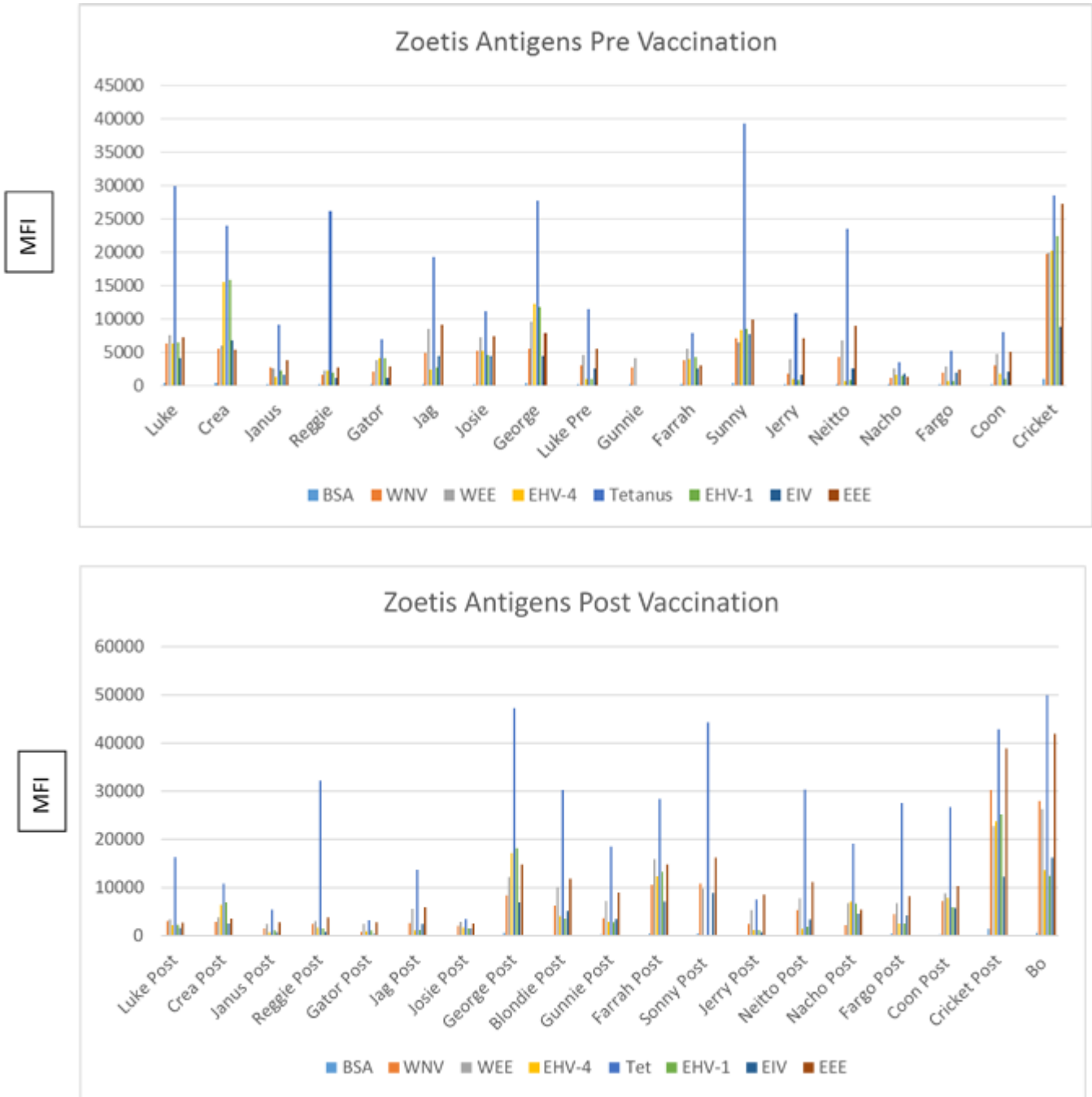
two separate plates.

Figure 2.3 – Graphical representation of all 7 antigens (WNV, WEE, EHV-4, EHV-1, EIV, and EEE) plus Bovine Serum Albumin pre and post-vaccination.
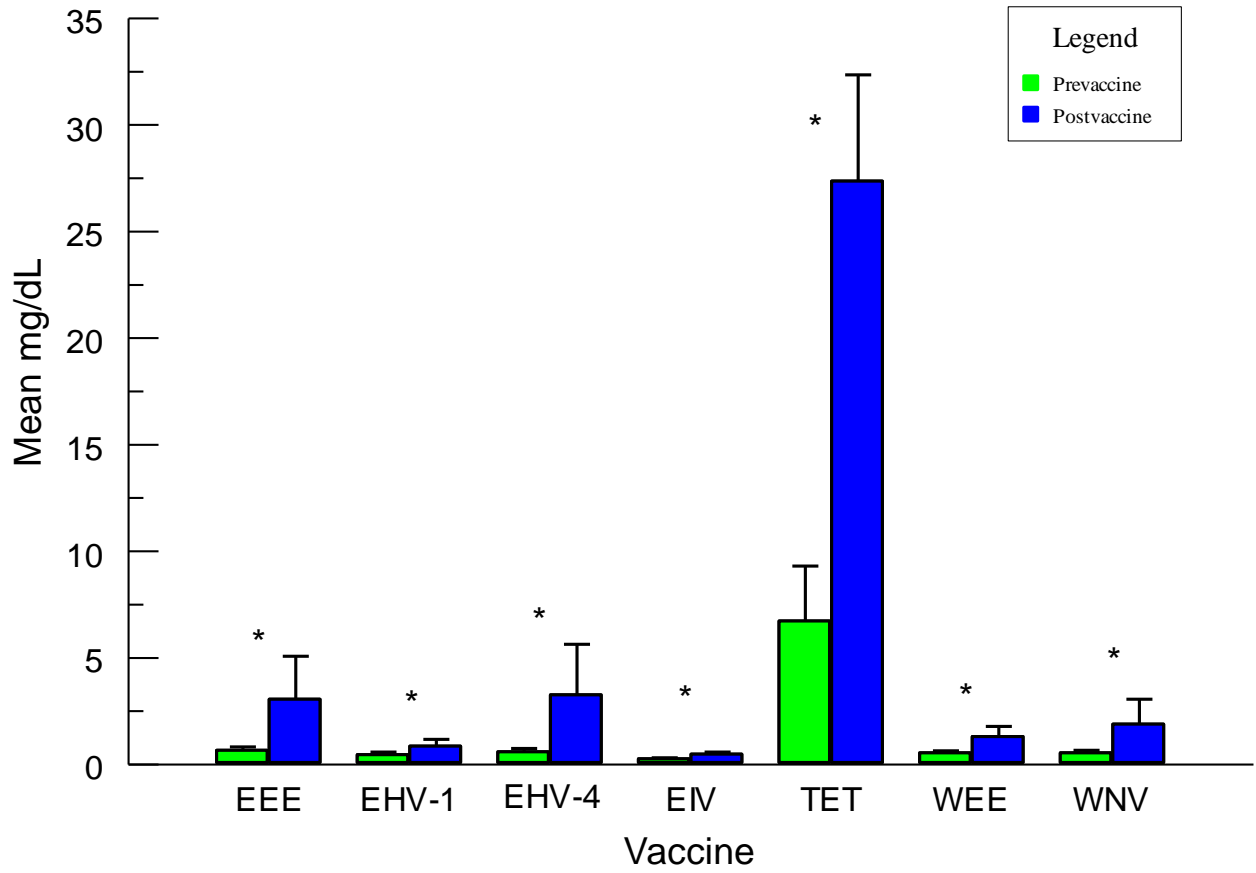
Figure 2.4 - Clinical representation using milligrams/deciliter units for all 7 vaccine antigen
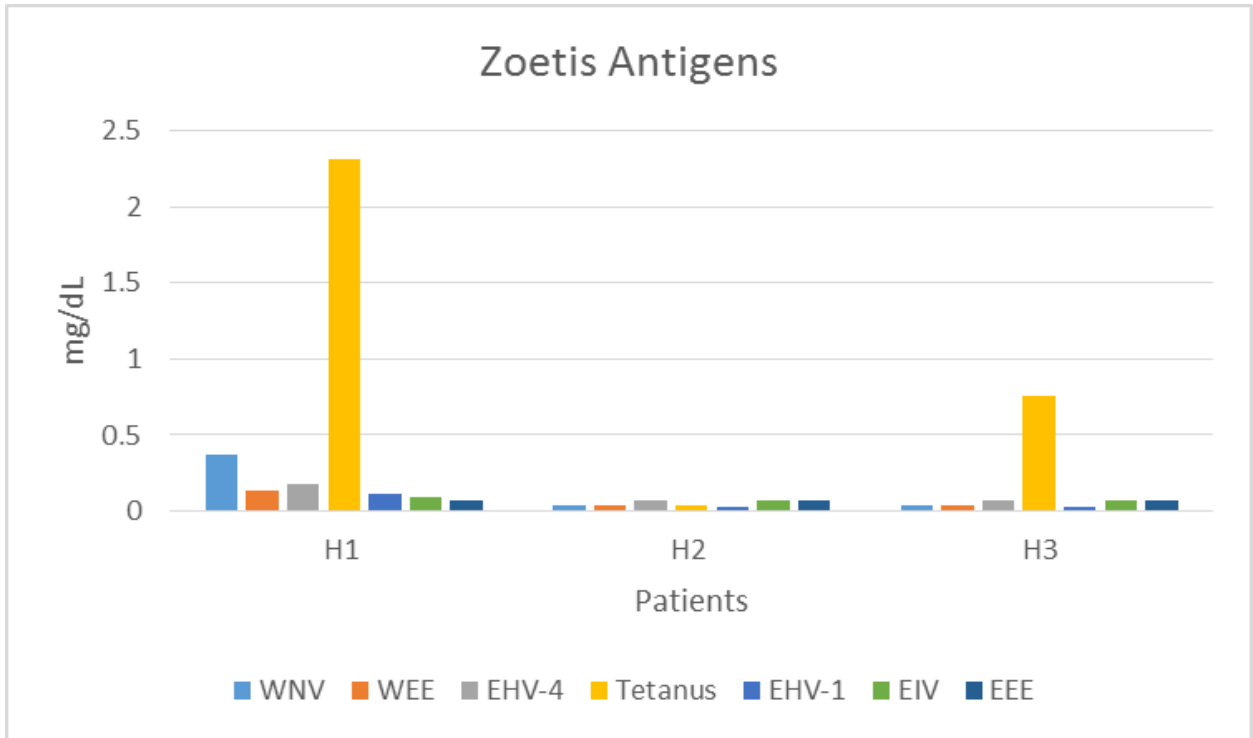
strains

Figure 2.5 – Graphical representation of one equine sample that had an un-known vaccine history and one equine sample that had a previous vaccine reaction.

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